

DESIGN, SYNTHESIS AND CHARACTERISATION OF A NOVEL TYPE II B-RAF PARADOX BREAKER INHIBITOR

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1	DESIGN, SYNTHESIS AND CHARACTERISATION OF A
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27 ABSTRACT

The mutation V600E in B-Raf leads to MAPK pathway activation, uncontrolled cell proliferation, 28 and tumorigenesis. ATP competitive type I B-Raf inhibitors, such as vemurafenib (1) and 29 30 PLX4720 (4) efficiently block the MAPK pathways in B-Raf mutant cells, however these 31 inhibitors induce conformational changes in the wild type B-Raf (wtB-Raf) kinase domain leading to heterodimerisation with C-Raf, causing paradoxical hyperactivation of the MAPK pathway. 32 This unwanted activation may be avoided by another class of inhibitors (type II) which bind the 33 kinase in the DFG-out conformation, such as AZ628 (3) preventing heterodimerization. Here 34 35 we present a new B-Raf kinase domain inhibitor that represents a hybrid between 4 and 3. This 36 novel inhibitor borrows the hinge binding region from 4 and the back pocket binding moiety from 37 **3**. We designed, synthesized, determined its binding mode, performed activity/selectivity 38 studies, and molecular dynamics simulations in order to study the conformational effects 39 induced by this inhibitor on wt and V600E mutant B-Raf kinase. We discovered that the inhibitor 40 binds in a DFG-out/ α C-helix-in conformation, did not induce the aforementioned paradoxical 41 hyperactivation in the MAPK pathway, and it was active and highly selective for B-Raf. We 42 propose that this merging approach can be used to design a novel class of B-Raf inhibitors for 43 translational studies.

44 INTRODUCTION

Protein Kinases (PK) constitute the third largest protein families in eukaryotes and are 45 responsible for regulating most cellular signaling processes. Aberrant expression of PKs has 46 often been linked to cancer development, making PKs interesting targets for drug discovery 47 and development, and consequently, as of 2020, 68 small-molecule PK inhibitors (PKI) have 48 been approved by the FDA (Bournez et al., 2020; Ferguson and Gray, 2018; Roskoski, 2019). 49 50 B-Raf, a serine/threonine PK, is an important component of the RAS/Raf/MEK/ERK signal 51 transduction pathway (mitogen-activated protein kinase or MAPK signaling cascade) and it is 52 a key regulator of cell proliferation and survival (Robinson and Cobb, 1997). Binding of small 53 GTPases of the RAS family to B-Raf induce structural changes that trigger activation of B-Raf 54 and the MAPK signaling cascade which involves MEK kinases and ERK that phosphorylate 55 diverse substrates including transcription factors (Kolch, 2000; Morrison and Cutler, 1997). The 56 B-Raf V600E missense mutation is one of the most commonly observed mutations in 57 melanoma (Fiskus and Mitsiades, 2016; Holderfield et al., 2014). B-Raf V600E causes B-Raf 58 to signal independently from its upstream signaling partners leading to activation of MAPK signaling resulting in increased cell proliferation and tumorigenesis (Cope et al., 2019; Davies 59 et al., 2002). Potent B-Raf inhibitors such as vemurafenib (Zelboraf®; 1 in Chart 1) (Bollag et 60 al., 2010) and dabrafenib (Tafinlar®; 2 in Chart 1) (Rheault et al., 2013) are highly effective for 61 treatment of patients with metastatic melanoma harboring the B-Raf V600E mutation (Carles 62 63 et al, 2018).



However, the therapeutic effects of these inhibitors are challenged by rapid development of 67 68 drug resistance, and occurrence of secondary tumors such as cutaneous squamous cell 69 carcinoma and keratoacanthoma (Alcalá and Flaherty, 2012; Anforth et al., 2013; Solit and 70 Rosen, 2014). By targeting and stabilizing the active conformation of the wild-type B-Raf (^{wt}B-71 Raf), these drugs paradoxically activate MAPK pathway giving rise to tumor development in 72 healthy tissue. Hence, these ATP-competitive inhibitors can either inhibit or (paradoxically) 73 activate the MAPK pathway depending on whether V600E mutant or ^{wt}B-Raf, is predominantly 74 expressed (Carnahan et al., 2010; Hatzivassiliou et al., 2010; Poulikakos et al., 2010). In fact, this paradoxical activation was already reported in 1999 (Hall-Jackson et al., 1999). 75

During the past few years, this paradoxical activation of MAPK pathway by ATP-competitive B-Raf inhibitors (paradox inducers; PI) has presented a novel and compelling challenge for second generation B-Raf inhibitor development. Several mechanistic explanations have been put forward to explain this phenomenon. A few recent studies have allowed drawing a consistent picture of ^{wt}B-Raf inhibition by type I and II inhibitors targeting the active and inactive states of 81 the protein respectively, and subsequent events such as dimerization and MEK/ERK signaling 82 (paradoxical activation) (Peng et al., 2015; Yao et al., 2015; Zhang et al., 2015). In addition to B-Raf homodimers, the B-Raf/C-Raf heterodimers may also act as paradoxical MAPK 83 84 activators (Heidorn et al., 2010) and both 1 and 2 (Chart 1) are weak inhibitors of C-Raf in comparison to B-Raf. Therefore, it is unlikely that B-Raf/C-Raf heterodimers are effectively 85 inhibited by these drugs. Other studies have characterized the inhibitor binding mode and 86 monomer/dimer occupancy preference of many type II B-Raf inhibitors (for example BGB659, 87 88 AZ628, TAK-632, and BI882370), using their crystal structures in complex with B-Raf or B-89 Raf/C-Raf heterodimer, to gain useful insights into the mechanism of inhibition and the 90 paradoxical activation (Liu and Gray, 2006, Yao et al. 2016, Hatzivassiliou et al., 2010, Wang 91 and Kim, 2012, Nakamura et al., 2013; Okaniwa et al., 2013, Lavoie et al., 2013, Waizenegger 92 et al., 2016).

93

94 In 2015, Zhang et al. (Zhang et al., 2015) reported the so-called 'paradox breaker' (PB) 95 compounds which intriguingly are structurally very close to **1**. Importantly these inhibitors were not type II inhibitors, but they still did not cause paradoxical activation (Le et al., 2013; Zhang 96 97 et al., 2015). Briefly, the sulfonamide side chain present in the structure of **1**, was replaced by 98 an N-methyl, N-ethylsulfonylurea. Surprisingly, the crystal structures of the two complexes were 99 perfectly aligned, although there seemed to be some very subtle changes in the position of 100 Leu505, a residue positioned near the C-terminus of the α C-helix, which is an integral part of 101 the regulatory spine (R-spine) (Taylor and Kornev, 2011). Interestingly, when the 2,6-difluoro-102 phenylsulfonamide in dabrafenib (2) was replaced by an N-methyl, N-ethylsulfonylurea tail, the 103 resulting compound showed reduced ERK activation, providing a new, rational basis for the design of paradox breakers. In a follow up study, the same group (Yao et al., 2019) 104 demonstrated that the clinical candidate, PLX8394, only occupies one of the dimer active sites, 105

and disrupts B-Raf homo- and B-Raf-C-Raf heterodimers by very subtle interactions withLeu505.

In 2017, a novel selective pan-Raf inhibitor REDX05358 was reported by Redx Pharma (Mason 108 109 et al., 2017), which, in comparison to vemurafenib, led to a more sustained inhibition of the 110 MAPK pathway. It was reported to induce minimal paradoxical activation in ^{wt}B-Raf cells by inhibiting the MAPK signaling in both monomer and dimers with equal potencies. The crystal 111 structure for this compound is not yet available and therefore the structural features contributing 112 113 to paradox breaker properties are not known. In another recent study, Wang et al. (Wang et al., 114 2017) have reported a new type II inhibitor designed by fragment linking method which has low 115 toxicity and is highly selective and active for B-Raf V600E. However, in the absence of the 116 crystal structure, it is difficult to ascertain the details of the binding mode. As of last year, next-117 generation (dubbed as type IV) allosteric peptide B-Raf inhibitors have been introduced that 118 target the Raf dimerization interface, and in combination with ATP-competitive inhibitors also 119 prevent the paradoxical activation of the MAPK pathway (Beneker et al., 2019; Gunderwala et 120 al., 2019). For a relatively recent review of B-Raf inhibitors, see Agianian and Gavathiotis, 2018 121 (Agianian and Gavathiotis, 2018).

We analyzed four B-Raf kinase inhibitors, two paradoxical inducers (vemurafenib **1** and PLX4720 **4**; Chart 1) and two paradox breakers (P-0013 (**5**; Chart 1) and P-0012 (Ibrahim et al., 2009), to unravel the biological mechanism of inhibition of these compounds at a structural and cellular level (Arora et al., 2015; Di Michele et al., 2015). From these studies, we identified key structural features, in particular hydrogen bridges formed between gatekeeper T529 residue and the sulfonamide in **1** and **4**, which we hypothesized could combined with other structural effects contribute to the paradoxical activation effect.

As part of our ongoing study of the B-Raf system, and to further contribute to the intense interest in this field of research (Man et al., 2019; Wang et al., 2019; Yaeger and Corcoran, 2019, 131 Brummer and McInnes, 2020, Riegel and Rajalingam, 2020), here we focus on the design of 132 new paradoxical breaker inhibitor using structural information of both a type I and II kinase 133 inhibitors. We compared two inhibitors of B-Raf: 3 (type II DFG-out or type IIA DFG-out/ α C-134 helix in) and 4 (classified as type I since it binds in the DFG-in conformation or as type IIB since 135 the α C-helix is in an "out" conformation). Previously, Wenglowsky *et al.* (Karoulia et al., 2016; Wenglowsky et al., 2012, 2014) have shown that introduction of the sulfonamide side chain in 136 **3** changes the type II into a type I mode of binding in the B-Raf ATP active site. In order to keep 137 138 the physicochemical properties and 3-dimensional shape of the new hybrid compound similar 139 to **3**, we kept the 5-chloro-azaindole hinge region scaffold as present in **4** and the benzamide 140 side chain in **3** binding in the allosteric hydrophobic pocket (Scheme 1). With the substituted azaindole moiety in place, we were able to draw conclusions about the importance of the 141 142 replacement of the propylsulfonamide by a substituted benzamide for the selection of type I or 143 type II binding modes.

144 **Scheme 1**. Hybrids 16 and **6** from **3** and **4** 145



- 148
- 149 The superposition of the two crystal structures of 4 (PDB ID 3C4C) and 3 (PDB ID 4G9R) in B-
- 150 Raf highlighted the conserved structural elements present in the complex of the hybrid
- 151 compound **6** (Figure 1).
- 152 **Figure 1.** Superimposition of the B-Raf crystal structures of **3** (blue), **4** (yellow), **6** (magenta,

153 WT). Note the difference in the orientation of F595 for the DFG-in and DFG-out conformations 154



157

158 The interactions of the carboxylic acid group of Glu501, and the backbone NH of Asp594 with 159 the inhibitor potentially explain the selection of the type II folding. Docking studies of the hybrid 160 compound **6** with B-Raf were conducted in order to support our design hypothesis. Furthermore, 161 the synthesized B-Raf inhibitors were tested on a large panel of protein kinases to evaluate 162 their selectivity profile and were subsequently studied on two relevant human cell lines to 163 assess their paradoxical activation effects. Finally, the crystal structures of the hybrid compound 164 **6** in ^{wt}B-Raf and V600E mutant revealed a dimeric structure in which both ATP binding sites 165 were occupied.

166

167 **RESULTS AND DISCUSSION**

168 Synthesis of hybrid compound 6

Hybrid compound **6** was prepared according to the scheme below (Scheme 2), which was
modified from the original synthesis of **4** (Tsai et al, 2008).

171 **Scheme 2.** A. 2,6-difluoro-3-nitro-benzoylchloride, AlCl₃, CH₂Cl₂, reflux, 96h, Y 50%; b. H₂, 172 Pt/C-5%, thiophene, THF, rt, 72 h, Y 96%; c. 3-dimethylacetonitril-benzoylchloride, pyridine,

173 THF, rt, 16 h, 51%.

174



175

Briefly, Friedel-Crafts acylation of 5-chloro-1H-pyrrolo[2,3-b]pyridine I with benzoylchloride II
gave keto derivative III in 50% yield after HPLC purification. Hydrogenation of the nitro group
gave aniline IV in 96% yield, which was selectively acylated under standard conditions in 51%
yield.
Hybrid compound **6** was tested in the ScanMAX kinase assay panel from Eurofins Discovery,

181 together with **1**, **3-5**, and identified as a very potent inhibitor of both ^{wt}B-Raf and B-Raf(V600E),

182 but it was less selective than **3** and **4** (Table 1).

183

184 Activity and selectivity of 1, 3, 4, 5 and 6 (ScanMAX)

185 **Table 1.** Selected B-Raf inhibitors (Chart 1) with activity and selectivity data included

Compound	B-Raf IC50	B-Raf(V600E) IC50 [µM]	Selectivity @1 uM	Other notable actives >80% inhibition@1
	[µM]	- 000 [h]	(#kinase >50%	μM
			inhibition/# kinase tested)	
1	0.130	0.062	30/451; 14/103	MEK5, PDGFRB,
(Vemurafenib)				MEK4, KIT, FGR,
				Raf1
3 (AZ628)	0.0013	0.00085	28/103	PDGFRB, KIT,
				ABL1-nonphos.,
				LCK, CSF1R, p38-α,
				RET, RSK1, CSK,
				p38-β, FLT1, BLK,
				FLT4, VEGFR2,
				PDGFRA, LYN,
				Raf1, HCK, SRC,

				YES, MEK5, TXK,
				FYN
4 (PLX4720)	0.025	0.037	19/103	MEK5, PDGFR,
				MEK4, Raf1, KIT,
				FGR
5 PLX7683 (P-	0.69	1.41	22/103	MEK5, VRK2,
0013)				PDGFRB, BLK, FGR
6	0.056	0.048	38/103	KIT, BLK, CSF1R,
				FLT4, RET, MEK5,
				LCK, SRC, p38-α,
				MAP4K4, FLT3,
				FLT1, LYN,
				VEGFR2, FGR, p38-
				β , HCK, PDGFRA,
				TXK, FYN, Raf1,
				YES, ABL1-phos.,
				FGFR2, CDK11,
				FGFR1, BMX, CSK

186

187 Cell Viability and Downstream signaling

In order to investigate the effect of the B-Raf inhibitors in living cells, the human colon cancer cell line SW260 expressing ^{wt}B-Raf and the malignant melanoma A375 cell line expressing B-Raf(V600E) were treated with increasing concentrations of B-Raf inhibitors. The MTT assays (Figure 2; Table 2) showed that in the cell lines expressing B-Raf(V600E) all studied inhibitors resulted in inhibition of proliferation, with varying potencies, the hybrid compound **6** being only

193 weakly active. In SW260 expressing ^{wt}B-Raf, we observed a slight increase in proliferation at

high concentration of **1** and **5**, but clear cytotoxicity of **3**, and the hybrid compound **6**. **1** and **5**

195 show little toxicity in this cell line.

196 Figure 2. Treatment of malignant melanoma A375 cell line expressing B-Raf(V600E) (left

panel); and human colon cancer cell line SW260 (^{wt}B-Raf; right panel) in MTT assay, by
 increasing concentrations of inhibitors 1, 3, 5 and 6

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202

203 **Table 2:** Cytotoxicity as measured with an MTT assay for 4 selected compounds

	IC ₅₀ (µM)						
Compound	SW620	A375					
1	No inhibition, slight activation	0.088					
3	0.166 uM	0.042					
5	No inhibition, slight activation	0.560					
6	2.810 uM	~10					

204

The effect of the four B-Raf inhibitors on the MAPK signaling pathway was evaluated by measuring the phosphorylation of MEK and ERK. As expected, the treatment with all inhibitors led to reduced pMEK and pERK levels in cells expressing B-Raf(V600E) (Figures 2, 3). In cells

- 208 expressing ^{wt}B-Raf, **1** induced the pMEK and pERK levels due to paradoxical activation effects
- (Figures 2, 3) in agreement with the previous reports (Zhang et al., 2015). As expected, paradox 209
- breakers **3** and hybrid compound **6** showed little or no activation of ERK. 210
- 211 Figure 3. Western blot analyses of SW260 (expressing ^{wt}B-Raf) and A375 (expressing B-
- Raf(V600E)) cell-lines treated with the inhibitors studied in this work. Treatment with 3 and 212
- hvbrid compound 6 does not lead to paradoxical ERK activation 213
- 214



- 216
- 217

Binding modes of 6 in wild type and V600E B-Raf 218

From earlier studies by Wenglowsky et al., (Wenglowsky et al., 2011), it is known that the propyl-219 and phenyl sulfonamide derivatives both bind to a DFG-in conformation with concomitant 220 221 movement of the α C-helix. In case of the phenylsulfonamides, a reorientation of Phe596, part of DFG motif, is observed (PDB ID 3SKC). It is clear that the change from amide to sulfonamide 222 has a deciding impact on the binding mode. The amide in 3 is engaged in the canonical 223 224 hydrogen bond network with the backbone of Asp654 and the carboxylic acid of Glu501. To get insight into the binding mode of hybrid compound 6 we co-crystallized this inhibitor with wild 225 226 type and V600E mutant B-Raf. The crystal structures showed a dimeric assembly with both 227 ATP sites occupied by the inhibitor in agreement with the lack of MAPK activation observed in

- 228 cellular assays. Dimeric structures are frequently observed even for inhibitors that break B-Raf
- 229 dimer in cellular assays due to the high protein concentration used in crystallization experiments
- 230 (Thevakumaran et al., 2015).
- 231 Superimposition of both monomers of the dimer revealed DFG-out (type-II) binding mode in
- wild type as well as V600E mutant B-Raf (Figure 4). We observed however a number of striking
- 233 structural features by comparing the monomer structures. αC-helix was in an "in" position as
- indicated by formation of the canonical salt bridge between the VAIK motif lysine (K483) and
- 235 the α C glutamate (E501).

Figure 4. Structure of B-Raf in complex with 6. A) dimeric B-Raf in the crystal structure. B) Superimposition of the two monomers demonstrating different state of the activation segment, which is fully ordered in monomer A but disordered in B. C) Structural comparison with 4 reveals not only distinct DFG conformations, 'out' in this structure, and 'in' in 4 (pdb code 3C4C), but also slight difference in the α C-helix positions. Close-up details of the inhibitor binding sites in monomer A and B with 6 (D and E, respectively) and 4 (F).



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Despite the induced DFG-out conformation, the activation segment was well organized in chain
A of the monomer but was unstructured in chain B, this might be due to crystal contacts. In the
mutant, E600 was involved in a number of long-range polar interactions with the αC N-terminus

249 (Q493) but the residue was too distant to form efficient hydrogen bonds. In chain B the activation segment was oriented towards the solvent and it was unstructured after residue T599. 250 The DFG phenylalanine (F595) stacked end on against the aromatic ring system of the 251 252 pyrrolopyridine hinge binding motif which formed the expected ATP mimetic hydrogen bonds with the main chain carbonyl of Q530 and the amid nitrogen with C532. Differences were seen 253 between the interactions of the P-loop phenylalanine (F468) with the inhibitor. In chain A, the 254 P-loop phenylalanine was flipped into the active site causing a strong distortion of the P-loop 255 and the phenylalanine side chain was stabilized by aromatic stacking interaction with the 256 257 inhibitor. In chain B the P-loop F468 was extended in an active conformation interacting with 258 αC-helix. A similar P-loop conformation has been described for 4 (PDB ID: 3C4C). However, 4 259 assumes a type I binding mode stabilizing a displaced α C-helix that rotated around its axis 260 removing the conserved αC glutamate (E501) from the ATP site.

261 The sulfonamide in **4**, a paradox inducer complexed in the DFG-in form of B-Raf, was engaged 262 in hydrogen bond with the backbone of Phe595 and Gly596 and potentially with the side-chain 263 of Lys483, as observed in the PDB structure 3C4C but not in the PDB structure 4WO5. These changes in the hydrogen bond network are accompanied by a switch of the α C-helix from the 264 265 "in" state to the "out" state. A similar behavior has been observed for 1, for which the sulfonamide motif is linked to the main chain of Phe595 and Gly596 with an αC-helix-out 266 conformation. When replacing the sulfonamide motif by an amide in the hybrid compound 6, 267 268 the canonical hydrogen bond network described previously was restored, causing the α C-helix to assume an "in" conformation. However, based on the α C-helix conformations of other 269 270 inhibitors (Table 3), it is unlikely that the conformation of the α C-helix alone explained the paradoxical breaker effect of **3** and of our hybrid inhibitor. Indeed, we notice in Table 3, listing 271 the X-ray structures of B-Raf complexes, 3 PB inhibitors (PLX7904, PLX7922 and BI 882370) 272 induced an αC-helix-out conformation in B-Raf. On the other hand, the B-Raf structures 273

complexed with paradox inducer compounds may have an α C-helix with "in" or "out" conformation. One conclusion that emerged from structural information of B-Raf inhibitor complexes listed in Table 3 was that if the ligand harbors a sulfonamide or a sulfonyl urea motif, the α C-helix adopts an "out" conformation, regardless of whether the ligand is a paradox breaker or paradox inducer and whether the inhibitor induces DFG-in or a DFG-out conformation. Therefore, we studied the conformational dynamics of B-Raf in complex with these inhibitors using molecular dynamics (MD) simulations.

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282 **MD simulations of the human** ^{wt}B-Raf **inhibitor complexes**

400 ns of MD simulations were carried out on the three inhibitor complexes: B-Raf/**3**, B-Raf/**4** and B-Raf/**6**. The α C-helix RMSDs (Figure 5(a)) showed that each of the three simulations reached equilibrium around a RMSD of 2.5 Å with respect to their respective experimental structures. The per-residue RMSF profiles for the B-Raf kinase domain complexed with each of the three ligands are shown in Figure 5(b).

Figure 5. (a) Root Mean Squared Deviation (RMSD); and (b) Per residue Root Mean
 Squared Fluctuation (RMSF) during 400ns of MD simulation of B-Raf complexed with 3, 4 and
 6



We observed that the RMSF profiles for the B-Raf kinase domain complexed with **3** and with **6** were very similar. In comparison, the B-Raf kinase domain complexed with **4** was observed to be slightly more flexible than the two other systems, with a <RMSF>= 0.61 Å for B-Raf/**4** compared to a <RMSF>= 0.49 Å for B-Raf/**3** and B-Raf/**6**. The hydrogen bond network between

301 the ligand and the receptor was analysed for each system. The analyses of these data are 302 summarized in Figure 6. The H-bonds with the hinge residues, C532 for AZ628, Q530, and C532 for PLX4720 and the hybrid compound, were retained throughout the simulations. We 303 304 observe an additional H-bond with E501 for the two PB compounds which probably stabilizes 305 an "in" conformation of the α C-helix. We have previously reported interaction between type I PI inhibitors 1 and 4, and the gatekeeper residue T529 of B-Raf wild type over 100 ns MD 306 307 simulation, whereas this interaction was not observed with type I PB inhibitors (such as 5) 308 (Arora et al. 2015).



309 Figure 6. Bar chart showing the hydrogen bond occupancy between the analyzed inhibitors and important B-Raf residues, including the gatekeeper residue T529 310



314 In the current work, we also observe the interaction between T529 and 4 complexed with ^{wt}B-315 Raf over 400 ns MD simulations. It is noteworthy that this interaction with the gatekeeper residue was not observed in the two crystal structures of ^{wt}B-Raf complexed with **1** and **4**, 316 317 respectively PDB ID 30G7 and PDB ID 3C4C. It appeared that this interaction could be one of the features that could be discriminatory between type I paradoxical inducer (PI) and breaker (PB) inhibitors, although the structural and conformational basis of such discrimination is not clear. However, this argument may be strengthened by the observation that during our MD simulations this interaction was also absent in ^{wt}B-Raf complexed with **3**, a PB inhibitor which binds type IIB binding mode. Consequently, we expected, and indeed observed, that **6**, another PB inhibitor binding in type IIB binding mode, did not exhibit this interaction (Figure 6).

324	Table 3	Comparison	of binding mod	es of compounds	discussed in this paper
		-	5		

Compound	Binding	PI	PDB	Remark	Ref.		Chain A			Chain H	}
	mode	or				DFG	αC	occupied	DFG	αC-helix	occupied
		PB				(dF-	(dе-к Å)		(df-	(de-K Å)	
						hrd Å)	side chains		HRD	side chains	
							(de-k Å)		Å)	(dе-к Å)	
							Cα-Cα			Cα-Cα	
Vemurafenib (1)	IIB	PI	30G7	V600E	(Bollag et al., 2010)	in	out	yes	in	in	no
F Class O					2010)	(4.8)	(8.4)		(5.2)	(2.6)	
							(13.4)			(11.6)	
PLX4720 (4)	IIB	PI	3C4C	WT	(Tsai et al.,	in	out	yes	out	in	yes
F					2008)	(5.2)	(8.7)		(10.6)	(7.1)	
							(13.6)		(1010)	(12.1)	
			4WO5	WT	(Thevakumaran et al., 2015)	in	out	yes	in	Out	yes

						(5.9)	(10.9)		(5.6)	(~11.9)	
							(13.9)			(14.1)	
AZ628 (3)	IIA	PB	4G9R	V600E	(Wenglowsky et al., 2012)	out	in	yes	out	In	yes
						(10.3)	(3.5)		(10.4)	(3.4)	
							(11.3)			(11.3)	
Our hybrid (6)	IIA	PB	To be	WT	This work	out	in	yes	out	in	yes
F O			submitted			(10.3)	(2.865)		(10.4)	(2.726)	
							(11.038)			(11.070)	
			To be	V600E	This work	out	in	yes	out	in	yes
			submitted			(10.3)	(2.976)		(10.4)	(2.775)	
							(10.929)			(10.995)	
PLX7904	IIB	PB	4XV1	V600E	(Zhang et al., 2015)	in	out	yes	in	in	no
				X-ray	2013)	(4.7)	(8.082)		(5.6)	(4.117)	
				alternative			(13.245)			(11.499)	
н				for P-0013							
				(PLX7683)							

PLX5568	IIA	PI	4XV9	WT	(Zhang et al., 2015)	out	in/out?	yes	N/A	N/A	N/A
F O,					,	(10.7)	(8.750)				
$\begin{array}{c} CI \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ $							(13.326)				
Compound 18/2VX	IIB	N/A	4PP7	WT	(Wenglowsky	in	out	yes	in	out	yes
				X-ray	et al., 2014)	(4.8)	(11.715)		(4.8)	(11.215)	
				alternative			(13.701)			(13.510)	
				for cpd16							
Compound 4/0WP	IIA	N/A	4G9C	WT	(Wenglowsky et al. 2012)	out	in	yes	out	in	yes
					et al., 2012)	(10.3)	(3.0)		(10.3)	(3.0)	
							(11.1)			(11.1)	
Dabrafenib	IIB	PI	4XV2	V600E	(Zhang et al.,	in	out	yes	in	in	yes
					2015)	(4.2)	(7.5)		(4.4)	(9.7)	
							(12.6)			(12.3)	
			5CSW	WT	(Waizenegger et al., 2016)	in	out	yes	in	out	yes

F		(4.3)	(7.4)	(4.5)	(8.7)	
S S S S S S S S S S S S S S S S S S S			(12.6)		(12.9)	
H ₂ N N						

326 **Discussion**

In 2017, Wang *et al.* reported a series of pyrimidine scaffolds as potent pan-Raf inhibitors containing a benzamide functionality that engaged Glu501 and Asp594 in a hydrogen bonding network in a DFG-out orientation (Wang et al., 2017). The hybrid B-Raf inhibitor studied and characterized here utilized a similar scaffold and was observed to adopt a DFG-out conformation.

332 Many ATP-competitive B-Raf inhibitors have been shown to induce dimerization of the 333 BRAF kinase domains, where the protomer units are placed side-to-side and the dimer 334 interface located near the α C-helix (Hu et al., 2013; Rajakulendran et al., 2009). It has previously been shown that the relative position of α C-helix, as induced by the inhibitor, 335 336 is a factor in determining the occupancy of each protomer. For instance, a B-Raf inhibitor 337 such as TAK632, which stabilizes the α C-helix in "in" conformation, allows for the second 338 protomer to be also occupied by an inhibitor molecule (Nakamura et al., 2013; Okaniwa 339 et al., 2013). In comparison, **1** stabilizes the α C-helix in "out" conformation and does not allow for the other protomer in the B-Raf dimer to be occupied by an inhibitor molecule 340 (as seen in chain B of crystal structure 30G7) (Karoulia et al., 2016). Recent molecular 341 342 dynamics studies have suggested that an inter-protomer interaction of a dimer mediated 343 via the conserved N-terminal W450 residue (in B-Raf) resulting in a direct interaction of 344 the two R-spine motifs. Therefore, any dimer conformational change would likely involve 345 a disruption of this interaction of R-spines (Jambrina et al., 2016). At the level of paradox inducer and paradox breaker inhibitors, it has been suggested that the paradox inducer 346 347 inhibitors promote dimer-formation more than paradox breaker inhibitors, which leads to 348 enhanced activation. Essentially, paradox inducer inhibitors may hinder the

349 conformational changes, which may disrupt the dimer assembly, in the α C-helix region (Kondo et al., 2019; Tsai and Nussinov, 2018; Tse and Verkhivker, 2016), as has also 350 351 been noted by Jambrina et al. This further emphasizes the role of the conformational changes in the Raf dimer interface in paradoxical activation of the MAPK pathway 352 introduced by ATP-competitive inhibitors. However, a type II B-Raf inhibitor such as 353 354 AZ628 (3) also strongly introduces Raf-dimerisation, but does not trigger the paradoxical 355 activation characteristically caused by inhibitor-induced dimerisation (notably with type I 356 inhibitors as discussed above), as has been noted in the literature (Noeparast et al., 2018; 357 Savoia et al., 2019; Wang and Kim, 2012) and has been confirmed by the current study. This is attributed to sterically allowing occupancy of both protomers of the Raf dimer by 3 358 359 due to its longer residence time. This mechanism is consistent with that of another type II pan-Raf inhibitor TAK-632. The crystal structure of 6 with B-Raf dimer also revealed 360 occupancy in both protomers (Figure 7). Owing to the similarity in structures and binding 361 362 modes of **6** and **3**, this suggests a similar mechanism of inhibitor-induced dimerization and therefore a function of 6 as paradoxical breaker inhibitor. Binding of the hybrid 363 compound is essentially identical into WT and mutant protein. 364

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366

Given the important role of the conformation of the α C-helix in Raf activation and dimerization, more recent structural hypotheses about the involved residues are worth noting, specifically the role of the residue R506 which has been deemed to be crucial. It has been hypothesized that the orientation of the R506 residue alone can affect the B- Raf dimerization. It has also been reported that in the third generation PB inhibitors (such as PLX7904, PLX8934) the orientation of R506 is "out" along with rest of the α C-helix which prevents these inhibitors from promoting paradoxical activation (Karoulia et al., 2016). The orientation of R506 in some second generation (paradox inducer) B-Raf inhibitors such as vemurafenib (**1**) and dabrafenib is "in". The orientation of this residue R506 in our hybrid B-Raf (WT)/**6** has also been observed to be "out" which further points to a structural interpretation of its PB nature.

378

379 SIGNIFICANCE

The discovery of B-Raf inhibitor **6** represents a novel approach to design effective hybrid B-Raf inhibitors, which utilise the features of both type I and type II B-Raf inhibitors and do not paradoxically activate the MAPK pathway. This may represent a new class of inhibitors altogether and can help in enhancing our existing knowledge of mechanisms involved in B-Raf activation by inhibitors.

385

386 **EXPERIMENTAL PROCEDURES**

387

388 **Preparation of B-Raf complex models**

The crystal structure of the human ^{wt}B-Raf kinase domain (KD) complexed with the hybrid compound has been mutated at multiple sites in order to improve expression to allow crystallization. The starting B-Raf KD model was prepared using the Homology Modeling software Modeller 9.13 (Sali and Blundell, 1993). The sequence of the B-Raf KD was 393 obtained from its UniProt entry (B-Raf HUMAN, P15056, isoform 1). The crystal structure 394 of the B-Raf KD complexed with the hybrid compound was used as a template for building 395 the B-Raf KD model. This structure is in a DFG-out/ α C-helix-out conformation. The cocrystallized ligand in the template-binding site and the interacting water molecules were 396 397 constraint for induced fit for the generation of five models. The same protocol was used 398 for the generation of initial models for the human ^{wt}B-Raf KD complexed with AZ628 and 399 PLX4720. The crystal structures used as template for the building of these two other 400 models were the structure with PDB ID 4G9R and 3C4C respectively.

401

402 Molecular Dynamics Simulations

403 Molecular dynamics (MD) simulations were carried out on the B-Raf-inhibitor complex models that were obtained as described in the previous section. All simulations were 404 carried out using the Amber14 suite (Case et al., 2014) with the Amber12SB force field 405 406 parameters. The parameter and coordinate files for the inhibitors were prepared using the antechamber utility (Wang et al., 2006) of AmberTools14. GAFF force field parameters 407 (Wang et al., 2004) were used for the inhibitors, and the partial charges were calculated 408 409 using the AM1-BCC method. The topology files for the protein-inhibitor complexes were 410 prepared using the tleap utility of AmberTools14. All the systems were then solvated using 411 a TIP3P solvent box of 10 Å radii from protein surface counter-ions were added to 412 neutralize the system reaching a ionic strength of 0.15 M. In order to remove any possible structural artifacts resulting from model building, before running the MD simulations, the 413 414 solvated systems were subjected to minimization. This was performed in three steps. In 415 the first step, positional restraints on the position of the solute molecules were applied,

and only the solvent and ion molecules were allowed to minimize for 100 steps using the 416 417 steepest descent method followed by 2000 steps using the conjugate gradient method. 418 In the second step, positional restraints were applied on the atoms of the solvent molecules leaving the solute molecule free to minimize during 100 steps using steepest 419 420 descent algorithm followed by 2000 steps using the gradient conjugate methods. In the 421 third step, the entire system was subjected to minimization for 100 steps using the 422 steepest descent method followed by 2000 steps using the conjugate gradient method. 423 Following the minimization step, the systems were heated from 0 to 300 K using a 424 Langevin thermostat, at a constant volume, for 20 ps with time step of 2 fs. A weak restraint of 10 kcal/(mol Å²) was applied on the solute during this run. Once the system 425 426 was heated to 300 K, the solute restraints were gradually removed during 10 ps at a 427 constant volume, and the systems were then equilibrated over a period of 400 ps with a 428 time step of 2 fs under constant pressure conditions in order to relax the solvent density. 429 Finally, the production runs were carried out using the NPT ensemble for 400 ns with a nonbonded cut off of 10 Å and time steps of 2 fs at 300 K. During the equilibration and 430 production steps, bonds involving hydrogens were constrained using the SHAKE 431 432 algorithm. Long range electrostatic interactions were treated using the particle mesh 433 Ewald (PME) method (Walker et al., 2008). All the simulations were carried out using the 434 PMEMD module of the Amber14 suite, which is the reimplementation of Sander utility, 435 with improved performance on a GPU cluster.

436 Chemistry

437 Detailed experimental procedures can be found in the Supplementary material.

- 438
- 29

439

440 **Crystallography**

441 **Crystallography and structure analysis**

Recombinant ^{wt}B-Raf and B-Raf(V600E), both harboring 16 surface mutations, were 442 443 expressed and purified as described previously (Thevakumaran et al., 2015; Tsai et al., 444 2008). Purified proteins in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP and 5% 445 glycerol at 10-12 mg/ml were mixed with **6** at 2.5-fold molar excess, and the complexes were crystallized using sitting drop vapour diffusion method at 20 °C and various 446 447 conditions as summarized in the table 4. Diffraction data collected at Diamond Light Source, beamline I03 using wavelength of 0.97624 Å were processed and scaled with 448 Mosflm (Powell et al., 2013) and Scala, (Evans, 2006) respectively. Molecular 449 450 replacement was performed using Phaser (McCoy et al., 2007)

and the coordinates of published B-Raf structure (Thevakumaran et al., 2015). Iterative
cycles of model rebuilding alternated with structure refinement were performed in COOT
(Emsley et al., 2010) and REFMAC (Murshudov et al., 2011) respectively. The final
models were verified for their geometric correctness using MolProbity (Chen et al., 2010).
The data collection and refinement statistics are summarized in the table 4.

456 **Table 4**: Description of the final crystallographic models

Complex	^{wt} B-Raf/6	B-Raf(V600E)/6
PDB accession code	XXXX	XXXX
Data Collection		
Resolution ^a (Å)	57.45-1.65	74.55-1.65

	(1.74-1.65)	(1.74-1.65)
Spacegroup	<i>P</i> 2 ₁ 2 ₁ 2 ₁	P212121
Cell dimensions	<i>a</i> = 49.1, <i>b</i> = 97.6, <i>c</i> =	a = 49.3, b = 97.7, c =
	114.9 Å	115.3 Å
	$\alpha = \gamma = \beta = 90.0^{\circ}$	$\alpha = \gamma = \beta = 90.0^{\circ}$
No. unique reflections ^a	66,629 (9,169)	67,419 (9,443)
Completeness ^a (%)	99.1 (95.2)	99.4 (97.3)
l/σl ^a	21.2 (9.4)	19.3 (6.4)
R _{merge} ^a (%)	0.066 (0.166)	0.067 (0.245)
Redundancy ^a	8.4 (6.8)	8.1 (6.5)
Refinement		
No. atoms in refinement	4,345/68/574	4,306/68/574
(P/L/O) ^b		
B factor (P/L/O) ^b (Å ²)	17/11/27	17/11/29
R _{fact} (%)	15.4	15.2
R _{free} (%)	17.1	17.5
rms deviation bond ^c (Å)	0.016	0.016
rms deviation angle ^c (°)	1.6	1.6
Molprobity Ramachandran		
Favour (%)	96.79	97.16
Outlier (%)	0	0
Crystallization conditions	19% PEG3350, 0.1M	13% PEG3350, 0.2M

bis-tris-propane pH 7.0,	sodium nitrate, 5%
0.2M sodium bromide,	ethylene glycol
10% ethylene glycol	

^a Values in brackets show the statistics for the highest resolution shells.

458 ^b P/L/O indicate protein, ligand molecules presented in the active sites, and other (water

- 459 and solvent molecules), respectively.
- 460 ^c rms indicates root-mean-square.
- 461

462 Activity Assays

463 Cell Viability

464 SW620 and A375 cells were obtained from ATCC. Cell viability assays (MTT) were carried

out as described by Mosmann (Mosmann 1983). In brief, cells were seeded at 4000 and

466 1000 per well (96 well format), respectively, and treated with compounds the next day for

467 96 h. The compounds' impact on cell viability was analysed via the MTT assay.

468

469 Immunoblotting

SW620 and A375 cells were seeded at 750 000 and 300 000 cells per well (six well format) and 48 h later (75% confluence) treated with increasing compound concentrations for either 1 or 4 h. Cells were lysed in lysis buffer (10 mM Tris-HCL, 1% SDS, and 20 mM Na3VO4) and 10–25 µg of proteins from each sample were loaded, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated with the indicated primary antibodies and analyzed by the Odyssey detection system from LI-COR. Used primary antibodies for MEK, phospho-MEK, and phospho-ERK were purchased
from Cell Signaling, B-Raf from Santa Cruz, and actin from Sigma. Corresponding
secondary antibodies were used from Rockland and Invitrogen.

479

480

481 ACCESSION NUMBERS

The Protein Data Bank accession number for the hybrid compound **5**-bound to ^{wt}B-Raf
and V600E mutant structures reported in this paper is XXXX and XXXX respectively.

484

485

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501

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506 Supplementary materials

- 507 Chemistry
- 508 General
- 509 1H NMR spectra were recorded with Bruker Avance DPX 400 and 360 spectrometers, and
- 510 chemical shifts (δ) are expressed in parts per million (ppm) with TMS as internal standard. For
- all tested and final compounds where no analytical purity is mentioned, compounds were
- 512 confirmed >95% pure via HPLC methods.
- 513 Compounds 1, 3, and 4 were obtained from SelleckChem, and compound 5 was synthesized
- according to the procedure in WO 2012109075.
- 515

516 Preparation of hybrid compound 6



517

518 2,6-Difluoro-3-nitro benzoyl chloride

519 DMF (0.19 ml, 2.4 mmol) was added to a solution of 2,6-difluoro-3-nitro-benzoic acid (7.0 g,

520 34.5 mmol) in thionyl chloride (70 ml) at room temperature. The mixture was stirred and heated

521 to reflux overnight ($\pm 80^{\circ}$ C; SOCl₂ T_b=74.6°C). Excess of thionyl chloride was removed under

reduced pressure. 2,6-Difluoro-3-nitro benzoyl chloride was not isolated and directly used for thenext step.

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526 (5-Chloro-1H-pyrrolo[2,3-b]pyridin-3-yl)-(2,6-difluoro-3-nitro-phenyl)methanone (II):

527 Aluminum chloride (24.0 g, 180 mmol) was dissolved in DCM (105 ml) under an atmosphere of

528 nitrogen below 5°C. I (3.4 g, 22.4 mmol) in DCM (52.5 ml) was added and the reaction mixture

529 was stirred for 1 hour. 2,6-Difluoro-3-nitro benzoyl chloride (7.0 g, 31.6 mmol) in DCM (52.5

530 ml) was added at 0°C dropwise. The mixture was left stirring for six days at 45°C. After six days 531 the mixture was poured in aqueous HCl (1N) solution (300 ml) yielding compound II (4.83 g, 532 91%) as a white yellowish precipitate, which was used in the next step without further 533 purification. Additional II was isolated from the organic layer and further purified by Prep 534 HPLC (Stationary phase: RP Vydac Denali C18 - 10µm, 200g, 5cm, Mobile phase: 0.25% 535 NH_4HCO_3 solution in water, CH3CN) yielding another fraction of II (480 mg) as a white yellowish solid. ¹H NMR (360 MHz, DMSO-*d*₆) δ ppm 8.43 (s, 2 H), 8.18 (s, 1 H), 6.84 - 7.00 536 537 (m, 2 H), 5.24 (s, 2 H). MS (ESI) $[M-H]^{-} = 335.99$ and 335.92 found.





539 (3-Amino-2,6-difluoro-phenyl)-(5-chloro-1H-pyrrolo[2,3-b]pyridin-3-yl)methanone (III): To 540 II (1.0 g, 2.9 mmol) in THF (50 ml) was added Pt/C-5% (200 mg) and a 4% solution of 541 thiophene in diisopropyl ether (0.5 ml). Hydrogen was inserted in the reaction mixture and left 542 stirring for three days. The mixture was filtered over decalite and concentrated under reduced 543 pressure yielding III (923 mg, 93%) as a white yellowish powder. 1H NMR (360 MHz, DMSO-544 d_6) δ ppm 5.24 (s, 2 H) 6.82 - 7.04 (m, 2 H) 8.10 - 8.27 (m, 2 H) 8.43 (s, 2 H). MS (ESI) [M-H]⁻ 545 = 306.02 and 305.92 found. 546

547



549 N-[3-(5-chloro-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluoro-phenyl]-3-(1-cyano-1-

- 550 methyl-ethyl)benzamide (6)
- 551 To a solution of **III** (0.20 g, 0.65 mmol) in anhydrous THF, pyridine (0.5 mL, 6.5 mmol) is
- added, followed by 3-dimethylacetonitril-benzoylchloride (0.15 g, 0.72 mmol). After stirring
- 553 overnight at room temperature under a nitrogen atmosphere, the reaction mixture is concentrated,
- and the residue is purified by Prep HPLC (Stationary phase: Uptisphere C18 ODB 10μm, 200g,
- 555 5cm), Mobile phase: 0.25% NH4HCO3 solution in water, CH3CN), yielding the title compound
- (0.16 g, 0.33 mmol, 51% yield) as a solid white material.
- ¹H NMR (360 MHz, DMSO-*d*₆) δ ppm 13.17 (br s, 1 H), 10.41 (s, 1 H), 8.49 (d, *J*=2.4 Hz, 1 H),
- 558 8.46 (d, J=2.4 Hz, 1 H), 8.26 (s, 1 H), 8.11 (t, J=1.8 Hz, 1 H), 7.97 (d, J=7.7 Hz, 1 H), 7.75 -
- 559 7.85 (m, 2 H), 7.62 (t, J=7.8 Hz, 1 H), 7.35 (t, J=8.8 Hz, 1 H), 1.75 (s, 6 H) MS (ESI) [M-H]⁻ =
- 560 306.02 and 305.92 found.

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